Cellular Distribution of the P2X₄ ATP Receptor mRNA in the Brain and Non-Neuronal Organs of Rats*

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Received September 13, 1996

Summary. RNA blot analysis has shown that an ionotropic ATP receptor P2X₄ is widely distributed, as compared with other P2X subtypes. Cellular distribution of the P2X₄ ATP receptor mRNA was investigated by *in situ* hybridization technique in the brain and non-neuronal organs. In the brain, transcripts of the P2X₄ were widely distributed in various regions, with the highest signals in the cerebellar Purkinje cells and granule cells. In other organs, intense and selective expression was detected in seminiferous tubules of the testis and intestinal crypts of the colon, suggesting an involvement of ATP in spermatogenesis and intestinal secretion. A low but significant level of the P2X₄ mRNA was detected in the adenohypophysis and the cortex of the adrenal gland. The tissue- or cell-specific expression of the P2X₄ mRNA is of importance to understand the functional roles of the purinergic transmission system.

Adenosine-5'-triphosphate (ATP) is a bioactive substance present in vesicular and granular components of neurons and paraneurons (FUJITA et al., 1988). Neurons containing ATP were originally documented in the autonomic nervous system of the gut wall, and designated as purinergic neurons (BURNSTOCK, 1990). In paraneurons, which are endocrine in nature, ATP is co-stored in secretory granules with amines, peptides and carrier proteins such as chromogranins, and co-released with them (FUJITA et al., 1988). Although purinergic neurons and paraneurons have been suggested to be widely distributed throughout the body, their localization and structure as well as the functional significance of ATP remains unclear, due partially to the lack of specific staining methods.

ATP, an excitatory neurotransmitter, exerts its effects by binding to purinoreceptors on the cell surface (BURNSTOCK, 1990). The purinoreceptors are pharmacologically classified into P1-purinoreceptor (adenosine as the endogenous ligands) and P2-purinoreceptor (ATP and ADP as the endogenous ligands). The latter is further classified into an ionotropic receptor (P2X) and G protein-coupled receptor (P2Y). ATP mediates fast synaptic transmission through the P2X receptor from neuron to neuron and from neuron to effector cell, such as the smooth muscle. Three subtypes of the P2X receptor have been so far identified and termed P2X₁, P2X₂, and P2X₃. *In situ* hybridization has shown their restricted expressions in certain tissues: the P2X₁ in the smooth muscle of the vas deferens, urinary bladder and arteries (VALERA et al., 1994); P2X₂ in the pheochromocytoma PC12 cells and superior cervical ganglion (BRAKE et al., 1994); and P2X₃ in the dorsal root ganglia (CHEN et al., 1995; LEWIS et al., 1995). Recently, a fourth member of the P2X receptor family, P2X₄, has been identified by several research groups (BO et al., 1995; BUELL et al., 1996; SÉGUÉLA et al., 1996; SOTO et al., 1996; WANG et al., 1996). Rat P2X₄ is a protein consisting of 388 amino acid residues, which shares 50%, 49%, and 47% sequence identities with P2X₁, P2X₂, and P2X₃, respectively (WANG et al., 1996). In contrast to other P2X subtypes, RNA blot analysis of the P2X₄ receptor exhibited its widespread distribution in various organs including the brain. Therefore, it is expected that the P2X₄ receptor may be responsible for the purinergic transmission system shared by a variety of cells.

In the present study, we employed the *in situ*

*This study was supported in part by Scientific Research Grant from the Ministry of Education, Science and Culture, Japan.

hybridization technique to determine cellular localization of the P2X₄ mRNA in the brain and non-neuronal organs.

MATERIALS AND METHODS

Three non-overlapping antisense oligonucleotides were used for *in situ* hybridization, which are complementary to nucleotide residues 1149 to 1194, 1248 to 1292 and 1402 to 1446 of the rat P2X₄ cDNA (WANG et al., 1996). These oligonucleotides were labeled with ³³P-dATP, using terminal deoxyribonucleotidyl transferase (BRL, Gaithersburg, MD) at a specific activity of 0.5 × 10⁹ dpm/μg DNA.

Male Wistar rats, weighing approximately 200 g, were used. Under pentobarbital anesthesia at a lethal dose, the brain was freshly removed from the skull and frozen in powdered dry ice. Cryostat sections, 20 μm in thickness, were prepared and mounted on glass slides precoated with 3-aminopropyltriethoxysilane. They were fixed with 4% paraformaldehyde for 10 min and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). Some animals were perfused through the aorta with a physiological saline solution followed by either 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or Bouin's fluid. The hypophysis, adrenal gland, pancreas, colon, and testis were removed and immersed in the same fixative for an additional 6–12 h. The tissues were dehydrated through a graded series of ethanol and embedded in paraffin. Dewaxed paraffin sections were treated with 100 μg/ml of pepsin (DAKO, Denmark) in 0.2 N HCl at 37°C for 10 min.

The sections were prehybridized for 1 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 7.5), 4 × SSC (1 × SSC; 150 mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% sodium dodecyl sulfate (SDS), 200 μg/ml tRNA, 1 mM EDTA, and 10% dextran sulfate. Hybridization was performed at 42°C 10 h in the prehybridization buffer supplemented with 10,000 cpm/μl of ³³P-labeled oligonucleotide probes. The slides were washed at room temperature for 20 min in 2 × SSC containing 0.1% sarkosyl and twice at 55°C for 40 min in 0.1 × SSC containing 0.1% sarkosyl. The sections were either exposed to Hybema-β max (Amersham, Buckinghamshire, England) for 3 weeks, or dipped in Kodak NTB2 nuclear track emulsion and exposed for 2 months.

RESULTS

In situ hybridization analysis using three non-overlapping antisense oligonucleotide probes exhibited consistent labelings in the brain and other organs. The specificity of hybridization was also confirmed by the disappearance of the signals when an excess dose of cold oligonucleotides was added into the hybridization fluid (Figs. 2, 3). Analysis on horizontal brain sections revealed a predominant expression of the P2X₄ mRNA in the cerebellum (Fig. 1). In the cerebellum, intense hybridization signals were limited to the granule cells and Purkinje cells (Fig. 5). Although the P2X₄ mRNA was also expressed in various brain regions, such as the hippocampus and olfactory bulb, the signal intensity was considerably weaker when compared with that in the cerebellum (Fig. 1).

In situ hybridization staining of the non-neuronal organs showed high levels of the P2X₄ mRNA in the testis. The labeling was found in a subset of seminiferous tubules which were intermingled with hybridization-negative tubules (Fig. 2). Observation at a higher magnification revealed that spermatids and occasionally immature sperms expressed the P2X₄ mRNA, but not spermatogonia or primary spermatocytes (Fig. 6).

Another intense labeling was found diffusely in the crypt epithelium of the colon (Figs. 4, 7), where a denser accumulation of silver grains was found in the basal portion of epithelial cells. In contrast, the muscle layer—including the submucous and myenteric nerve plexuses—was free of the labeling.

Low but significant levels of the transcripts were found diffusely in the endocrine and exocrine glands. In the hypophysis, the P2X₄ mRNA was more abundant in the adenohypophysis (anterior lobe) than in the posterior and intermediate lobes (Fig. 8). The cortex and medulla of the adrenal gland were both labeled moderately, with slightly higher signals in the zona granulosa. Pancreatic tissue showed a faint but specific labeling throughout the tissue, but no particular labeling was recognizable in the islets.

DISCUSSION

The cDNA of the P2X₄ receptor was cloned from the rat hippocampus (BO et al., 1995), superior cervical ganglion (BUELL et al., 1996), brain (SÉGUÉLA et al., 1996; SOTO et al., 1996) and pancreatic islets (WANG et al., 1996). In prospects of the rich existence of the P2X₄ mRNA in the brain, some researchers have

Fig. 1. A hybridized horizontal brain section showing expression of the P2X₄ ATP receptor mRNA. The brain section was exposed to an X-ray film. Intense signals are restricted to the cerebellum (*Cb*). Much lower levels of the P2X₄ mRNA expression are widely distributed in various regions of the cerebrum, including the hippocampus (*Hc*) and olfactory bulb (*OB*). *Cx* cerebral cortex, *CP* caudate putamen.

Figs. 2 and 3. Dark-field micrographs of paraffin sections from the testis. **Fig. 2.** The section was hybridized with a radiolabeled oligonucleotide probe for the P2X₄ mRNA. Intense signals are seen in a limited number of seminiferous tubules (*arrows*) which are intermingled with negative tubules. **Figs. 3.** The hybridization signals for the P2X₄ mRNA disappear after the addition of an excess dose of cold oligonucleotides into the hybridization fluid. $\times 13$

Fig. 4. A dark-field micrograph of a paraffin section obtained from the colon. Intense signals are found throughout the mucosal layer, in which numerous profiles of crypts (*Cr*) are visible. No significant accumulation of silver grains is found in the muscle layer (*Mu*). $\times 18$

examined its localization in the rat brain, using the *in situ* hybridization technique. BUELL et al. (1996) and COLLO et al. (1996) reported the intense expression of P2X₄ mRNA in various brain regions, including the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and pineal gland. On the other hand, SÉGUÉLA et al. (1996) showed its preferential expression in the cerebellar cortex, with much lower levels in other brain regions. In the non-neuronal organs, the widespread distribution of P2X₄ has been revealed mainly by RNA blot analysis. Rat P2X₄ has been shown to be expressed in the lung, thymus, adrenal gland, testis, vas deferens, and urinary bladder (BO et al., 1995). In addition to these organs, SOTO et al.

(1996) also detected strong signals for the P2X₄ mRNA in the heart, uterus, kidney, small intestine and trachea of the rat (SOTO et al., 1996). Furthermore, WANG et al. (1996) demonstrated its rich existence in endocrine glands as well as various hormone-secreting cell lines. However, information about the cellular localization of P2X₄ mRNA is more limited in the non-neuronal organs than in the brain.

In the present study, *in situ* hybridization analysis with ³³P-labeled oligonucleotides specific to the P2X₄ mRNA was applied to the brain and various peripheral organs. Specificity of the present hybridization was confirmed by identical labeling obtained with three non-overlapping probes and by the disappear-

Figs. 5-8. Legends on the opposite page.

ance of signals with the addition of cold probes. The most intense labeling in the brain was restricted to the cerebellar cortex, in agreement with the data from the RNA blot analysis (WANG et al., 1996). Furthermore, the present study confirmed the preferential expression of the P2X₄ mRNA in the cerebellar Purkinje cells and granule cells, as shown by SÉGUÉLA et al. (1996). We also confirmed its intense expression outside the brain. Among the peripheral organs, intense signals for the P2X₄ mRNA were found in the testis and colon, in which the abundant expression has been demonstrated by RNA blot analysis (WANG et al., 1996). Although we recognized a significant expression of P2X₄ mRNA in the pituitary gland and adrenal gland, the expression intensity in the endocrine organs including the pancreas was not thoroughly consistent with the findings from the RNA blot analysis (WANG et al., 1996).

Hybridization signals for the P2X₄ mRNA were most intense in the testis. Since purinergic excitatory transmission in the testis has not yet been ascertained (BURNSTOCK, 1990), the functional significance of ATP in the testis remains unclear. The distinct localization of the P2X₄ mRNA in the seminiferous epithelium suggests an important role for ATP in spermatogenesis, especially for spermatid differentiation into sperms. Interestingly, the testis is an organ with poor innervation. Therefore, we should consider a hormonal action for ATP, which is secreted from some endocrine paraneurons possibly including Leydig's interstitial cells which, at least partly, have been claimed to be of paraneuronal nature (DAVIDOFF et al., 1993).

BUELL et al. (1996) first reported the presence of the P2X₄ receptor mRNA in an epithelium (acinar cells of salivary glands), implying that a purinergic system is involved in the transmission from postganglionic sympathetic fibers to epithelia. The intense expression of the P2X₄ mRNA in the intestinal epithelium suggests the role of ATP in non-adrenergic transmission, since the intestinal secretion is believed to be regulated mainly by cholinergic and peptidergic neurons (FURNES and COSTA, 1987). There is considerable data suggesting the coexistence of ATP with acetylcholine and with various peptides in enteric neurons, ever since an early study by BURNSTOCK et al. (1970). As another important source of intestinal

ATP, we cannot neglect endocrine cells dispersed in the epithelium. ATP and other adenine nucleotides are believed to be constant constituents of the secretory granules of paraneurons, as shown in adrenal chromaffin cells and pancreatic B cells (FUJITA et al., 1988). The lack of the P2X₄ mRNA in the muscle layer is interesting, because another subtype of purinoreceptor (P2X₁) is richly expressed in smooth muscles of some organs (VALERA et al., 1994). In the pancreas, ATP has been shown morphologically and pharmacologically to be richly present in islet cells. LEITNER et al. (1975) demonstrated that, after the experimentally induced degranulation of islet cells, ATP is released into the blood at a constant ratio to insulin release. KUROMI et al. (1995) have suggested recently that ATP released from pancreatic B cells stimulates insulin secretion from B cells in an autocrine mode. However, we failed to confirm the existence of P2X₄ ATP receptors in islet cells. The present *in situ* hybridization instead revealed acinar cells as a target of ATP, released from islet cells and possibly from purinergic nerve fibers which are distributed in islets.

P2X₄ mRNA expression in epithelia also has been reported in the respiratory epithelium and uterine gland (BUELL et al., 1996; COLLO et al., 1996). These findings suggest that the P2X₄ is closely related to epithelial functions. There is also a possible involvement of the P2X₄ in apoptosis of intestinal epithelial cells. The connection of the P2X purinoreceptors with apoptosis is suggested by their sequence similarity with the RP-2, a gene selectively expressed in cells induced to die (OWENS et al., 1991; VALERA et al., 1994). In fact, ATP has been shown to induce cell death in thymocytes (ZHENG et al., 1991), hepatocytes (ZOETEWIJ et al., 1992) and several lymphoid cell lines. The intestinal epithelial cells have a short life span (2–3 days) and die by apoptosis. The rich existence of the P2X₄ mRNA in the intestinal epithelium reminds us of its possible involvement in regulating the cell death of enterocytes.

Extensive distribution of the pharmacologically identified ionotropic purinergic system suggests the existence of a variety of target cells. Although the distribution of the three subtypes of the P2-purinoreceptors, i.e., P2X₁, P2X₂ and P2X₃, was restricted to particular types of cells, the P2X₄ mRNA demon-

Figs. 5–8. Bright-field micrographs of paraffin sections showing signals for the P2X₄ mRNA. In the cerebellum (**Fig. 5**), intense signals are seen on the granule cells and dispersed Purkinje cells indicated by *arrows*. *Gr* granule layer, *Mo* molecular layer. Intense and selective labeling is found in spermatids of the seminiferous tubules (**Fig. 6**). In the colon (**Fig. 7**), heavy accumulation of silver grains is restricted to the crypt region (*Cr*), while the muscle layer (*Mu*) is negative in reaction. In the hypophysis (**Fig. 8**), a less intense level of mRNA is expressed in the anterior lobe (*AL*). *IL* intermediate lobe. Figs. 5 and 8: $\times 190$, Figs. 6 and 7: $\times 150$

strated its widespread distribution especially in the non-neuronal organs. Very recently, novel types of the P2X receptors (P2X₅ and P2X₆) have been cloned from celiac and superior cervical ganglia mRNAs, respectively (COLLO et al., 1996). The P2X₅ mRNA is expressed exclusively in the mesencephalic trigeminal nucleus other than the celiac ganglion, while the P2X₆ mRNA showed a broad localization in the brain and non-neuronal organs comparable to the P2X₄ mRNA. The P2X₄ and P2X₆ may be key molecules for both understanding the variety of functions of ATP as well as the distributions of ATP receptors.

Acknowledgements. The authors cordially thank Dr. Masahiko WATANABE, Department of Anatomy, Hokkaido University School of Medicine, for his kind suggestions and support throughout the study.

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